Zebrafish: An Emerging Model for Orthopedic Research

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ABSTRACT: Advances in next-generation sequencing have transformed our ability to identify genetic variants associated with clinical disorders of the musculoskeletal system. However, the means to functionally validate and analyze the physiological repercussions of genetic variation have lagged behind the rate of genetic discovery. The zebrafish provides an efficient model to leverage genetic analysis in an in vivo context. Its utility for orthopedic research is becoming evident in regard to both candidate gene validation as well as therapeutic discovery in tissues such as bone, tendon, muscle, and cartilage. With the development of new genetic and analytical tools to better assay aspects of skeletal tissue morphology, mineralization, composition, and biomechanics, researchers are emboldened to systematically approach how the skeleton develops and to identify the root causes, and potential treatments, of skeletal disease. © 2019 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 38:925–936, 2020

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Small animal models amenable to rapid-throughput biology are needed to accelerate the discovery of new treatments for clinical disorders of the musculoskeletal system. Complex, multi-cellular interactions are difficult to recapitulate in a dish. While such processes can be studied in animal models, ready-made mutant lines often do not exist (e.g., see section "Disease loci"). The zebrafish (*Danio rerio*) is a small, tropical freshwater fish that, by virtue of its unique experimental attributes (e.g., small size, low cost, genetic tractability, and optical transparency), has opened powerful avenues for biomedical research (including studies of development,[1,2] neuroscience,[3] regeneration,[4] and disease[5]) that are difficult in other vertebrate models. Such avenues include in vivo imaging of cell dynamics, and genetic and chemical screens. Moreover, zebrafish can be used as a pre-screening tool to prioritize more labor and cost-intensive studies that require de novo mutant mouse generation. The potential benefits of incorporating zebrafish into a research program must be weighed with limitations, including infrastructure costs (which vary depending on the institution), differences in zebrafish and human genetics and physiology, and the fact that many experimental approaches are still in their infancy, and thus remain to be rigorously validated. Indeed, the use of zebrafish to understand clinical disorders of the musculoskeletal system has only begun to be established. Here, we introduce the experimental advantages of the zebrafish, discuss its genetic and physiological similarities and differences to humans, and survey recent applications to musculoskeletal development and disease. This review elaborates on a workshop of the same name at the 2019 Orthopaedic Research Society Meeting, conducted by the authors, the purpose of which was to introduce emerging orthopedic research in zebrafish to facilitate cross-talk, establish foundations, and develop new models of clinical disorders.

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GENETICS

Genetic Similarity to Humans

A key criterion in the selection of an appropriate disease model is its genetic similarity to humans. Approximately, 71% of human protein-coding genes possess at least one zebrafish ortholog.[6] This is comparable to mouse, as ~80–90% of human protein-coding genes possess at least one ortholog in mouse (http://www.informatics.jax.org/homology.shtml). As zebrafish arose from a common ancestor that underwent an additional round of whole-genome duplication relative to mice and humans, zebrafish can have multiple co-orthologs for human genes (e.g., human *RUNX2* has two zebrafish co-orthologs, *runx2a* and *runx2b*). While this can complicate testing of gene function due to issues such as functional redundancy, such difficulties can be alleviated through simultaneous knockdown or knockout of co-orthologs.[1,7] In other cases, the maintenance of two copies of a gene in the zebrafish often is balanced by the partitioning function of a gene or subfunctionalization. As such, the retention of co-orthologs in the zebrafish often permits nuanced analysis of gene function in genes that might be lethal in mice. In mouse, analysis of many genes often requires combinatorial genetic techniques to provide conditional spatial or temporal regulation of gene function, whereas in the zebrafish simple genetic alterations can be studied (e.g., *Fgfr1*[8]). The often partitioned function of paralogues in the zebrafish also permits loss-of-function

analysis to the model the effect of more nuanced alleles such as regulatory shifts in gene function underlying many common skeletal pathologies. Such pathologies cannot easily be modeled with knockout strategies in the mouse and due to the complex anatomical nature of many skeletal disorders, often cannot be modeled in vitro even when allele-specific cell lines are constructed. A large number of skeletal disease models have been identified in zebrafish,[9–11] and this number is steadily increasing.

Techniques

The ease of forwarding genetics in zebrafish gives this model an advantage for unbiased discovery of mutant phenotypes. *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screens in zebrafish have uncovered a large number of variants relevant to fundamental aspects of skeletogenesis,[12–14] morphological evolution,[15] and human skeletal diseases.[16–20] Early examples involved the identification of a large collection of mutants with defects in formation of the jaw and branchial arches.[12–14] The same screen also identified mutations, specifically affecting the adult form.[21] The mutants identified in these early large-scale screens served as foundation for experimental analysis and were proof that not only could specific mechanisms of skeletal development be identified, but that mutation could be in genes homologous to human genes associated with skeletal diseases. Such screens often yield specifically defined point mutations, which provide nuanced changes in gene function that simple loss-of-function mutations or frameshifts cannot. Screens for dominant mutations affecting skeletogenesis, in which mutations often lead to dominant-negative properties as well as alleles with increased functions (hypermorphic or neomorphic alleles), are proving to be informative and useful in disease modeling. For example, recent screens have identified the dominant mutants closely mirroring collagenopathies and osteogenesis imperfecta (OI) (*col1a1a/b*, *col1a2*), Adams-Oliver syndrome (*dll4*), and hyperhidrotic ectodermal dysplasia (*edar*).[16] Finally, the Zebrafish Mutation Project,[22] which has phenotyped a large number of zebrafish mutant alleles and made them available to the community, demonstrates the feasibility of systematic genome-wide analysis.

In addition to forward genetics, zebrafish are also readily amenable to reverse genetics, that is, testing for phenotypic consequences following the targeted interference of gene function. The advent of TALEN-[23] and CRISPR-based gene editing has substantially expanded the means by which the scientific community can approach reverse genetics in zebrafish. For gene editing using CRISPR, administration of Cas9:gRNA ribonucleoproteins (RNPs) generates double-stranded breaks at defined loci. Errors in the non-homologous end joining (NHEJ) repair mechanism lead to insertions and deletions (indels) at the cut site, often leading to loss of function (e.g., due to non-sense-mediated decay triggered by a premature stop codon). Alternatively, multiple RNPs can be used to induce site-spanning deletions that delete promoter regions or entire gene loci, which may help reduce activation of compensatory pathways triggered by messenger RNA degradation.[24] Moreover, Cas9:gRNAs can be co-injected with a donor template which, following homology-directed repair (HDR), can result in precise gene edits. Because zebrafish develop externally, hundreds of embryos can be injected by a single user in one morning. This allows for the efficient creation of induced mutations or replacements at specific genetic loci. Screening phenotypes in injected G0 founder "crispant" animals can further enable rapid and cost-effective assessment of gene function.[1] In addition to alleviating the time and resources needed to breed alleles to homozygosity, G0 screens are also amenable to multiplexing strategies, in which multiple genes are targeted in the same animal. The ability to detect adult skeletal phenotypes in G0 zebrafish for genes associated with recessive forms of OI (*bmp1a* and *plod2*) was recently demonstrated.[25]

Zebrafish also provide a versatile system to test gene function through the use of transgenesis. This allows for stable or inducible (e.g., heat shock-induced) protein expression, or conditional gene targeting (e.g., Cre-mediated recombination). The Tol2 transposon system is commonly used for introducing transgenes. There exists a large, growing panel of zebrafish fluorescent reporter lines for cell types within the musculoskeletal system (Table 1). As zebrafish are also relatively transparent and develop externally, development can be easily observed in real-time. With these two attributes, use of transgenic reporters for particular cell types and proteins has provided an unmatched ability to visualize the dynamics of skeletal patterning and regeneration. These advantages also permit the visualization of cell behaviors in specific genetic contexts to gain a mechanistic understanding of disease etiology.

Because of their small size and low cost, zebrafish are also amenable to drug discovery via chemical screens. In such screens, large libraries of small molecules are tested to identify specific compounds that affect gene function or developmental processes. In a typical screen, zebrafish embryos/larvae are dispensed into 48- or 96-well plates, drugs are administered by adding them to the water, and phenotypes are assessed (e.g., via morphological, fluorescent, or behavioral readouts). This strategy can be adapted to adults.[40,41] The identification of dorsomorphin as a selective inhibitor of bone morphogenetic protein (BMP) type I receptors were discovered in a large zebrafish chemical screen and led to the development of analogs for the treatment of heterotopic ossification.[42] In another screen, phosphodiesterase (PDE) inhibitors were found to alter phenotypes in a zebrafish model of Duchenne muscular dystrophy.[43] See Wiley et al.[44] for a recent review of chemical screens in zebrafish.

Table 1. Transgenic Lines for Visualizing Cells Relevant to the Musculoskeletal System

Tissue/Cell Type	Transgene	References	Notes
Early neural crest and CNC-derived cartilages	*Tg*(*sox10:GFP*)	[26]	
Early neural crest and CNC-derived cartilages	*Tg*(*sox10-CreERt2*)	[27]	Tamoxifen-inducible Cre
Cartilage/chondrocytes	*Tg*(*col2a1:eGFP*)	[28,29]	
Cartilage/chondrocytes	*Tg*(*Col2a1aBAC:mcherry*)	[30]	
Cartilage/chondrocytes	*Tg*(*1.7c2a1a:mEGFP*)	[28]	Membrane-tagged EGFP
Tendon cells	*Tg*(*scx:mCherry*)	[31]	
Muscle cells	*Tg*(*-0.5unc45b:mCherry*)	[32]	
Bone/pre-osteoblasts	*Tg*(*runx2:GFP*)	[33]	
Bone/osteoblasts	*TgBAC*(*col10a1a:Citrine*)	[34]	
Bone/osteoblasts	*Tg*(*sp7:EGFP*)	[35]	
Bone/osteoblasts	*Tg*(*osx:GFP*)	[35]	
Bone/osteoblasts	*Tg*(*osx:CreERt2*)	[36]	Tamoxifen-inducible Cre
Bone/osteoblasts	*Tg*(*Ola.Sp7:NLS-GFP*)	[37]	Nuclear localization signal-tagged GFP
Bone/osteoblasts	*TgBAC*(*entpd5a:Citrine*)	[38]	
Bone/mature osteoblasts	*Tg*(*ocn:GFP*)	[36]	
Bone/osteoclasts	*Tg*(*ctsk:YFP*)	[39]	
Bone/osteoclasts	*Tg*(*ctsk:DsRed*)	Personal communication	

EGFP, enhanced green fluorescent protein.

FORMATION AND INTEGRATION OF THE ZEBRAFISH MUSCULOSKELETAL SYSTEM

Development and Patterning

Fully developed, the zebrafish skeleton comprises several functional groups including the cranial skeleton, axial skeleton, caudal skeleton, unpaired fins (dorsal, anal, and caudal fins), paired fins (pectoral and pelvic fins), and elasmoid scales (Fig. 1A–D). As in all vertebrates, the zebrafish cranial skeleton and its associated connective tissues, tendons, and ligaments, arise from the cranial neural crest; the fin skeletal elements arise from the lateral plate mesoderm, and the myosepta and axial skeleton from somitic paraxial mesoderm.[45–47] The cranial musculoskeletal system forms rapidly and can function by 5 days post-fertilization (dpf). The pectoral fin cartilage and muscles are also developing at this time. Although the axial skeleton does not form cartilage and bone until later stages, it has the same somitic compartments, sclerotome, syndetome,[48] and myotome, fated to become skeletal, tendon, and muscle tissues as in higher vertebrates. Prior to 5 dpf, the axial musculoskeletal structures primarily are composed of muscle and myosepta, a *scleraxis*-expressing myotendinous tissue that links the myomeres.[49,50] The bony elements form through direct/intramembranous ossification, or via cartilage or cartilage-like template (e.g., via perichondral or endochondral ossification).[51–54] The modes of ossification can differ in zebrafish and mammals in similar bones. For example, in mouse, the vertebrae form by endochondral ossification[55]; in zebrafish, vertebrae form by direct mineralization of the notochord sheath (perichordal ossification), without passing through a cartilaginous stage.[56] In some bones, osteoblasts and osteoclasts act in concert to model bone shape into adulthood.[57] Although uncommon, osteon-like structures in zebrafish have been reported for lateral ethmoid bone.[54] Notably, these structures contained solely one lamella and no osteocytes. Indeed, most skeletal elements in adult zebrafish skeletons are osteocytic and do not show osteons or hemiosteons indicative of human-like secondary remodeling. In vertebrae of adult zebrafish, osteocyte lacunar orientation shows a preferred orientation (Fig. 1E).[58] While the mechanosensing and remodeling characteristics of osteocytic bone in zebrafish remain to be fully understood, lacunae in zebrafish indicate smaller volumes with less numerous canaliculi compared with mice and humans.

Tendons are the tissue interface between the muscle and bone.[59] Concurrent to the skeletal development, transcripts of *scleraxis a* (*scxa*) are found in the forming tendon cells adjacent to the developing cartilage and muscle by two dpf. These cells aggregate and differentiate, turning on the expression of tendon matrix genes, *tenomodulin* (*tnmd*), *thrombospndin-4* (*tsp4b*), and type I collagen (*col1a1a*/*b*, *col1a2*).[45,50] As in mammals, initiation of the axial tendon program depends on signals from the muscle. Cranial and fin tendons form in the absence of muscle, but require muscle for tendon maintenance.[45,60] FGF and transforming growth factor-β (TGF-β) are also important for proper tendon formation,[45] and *cyp26b1* loss of function

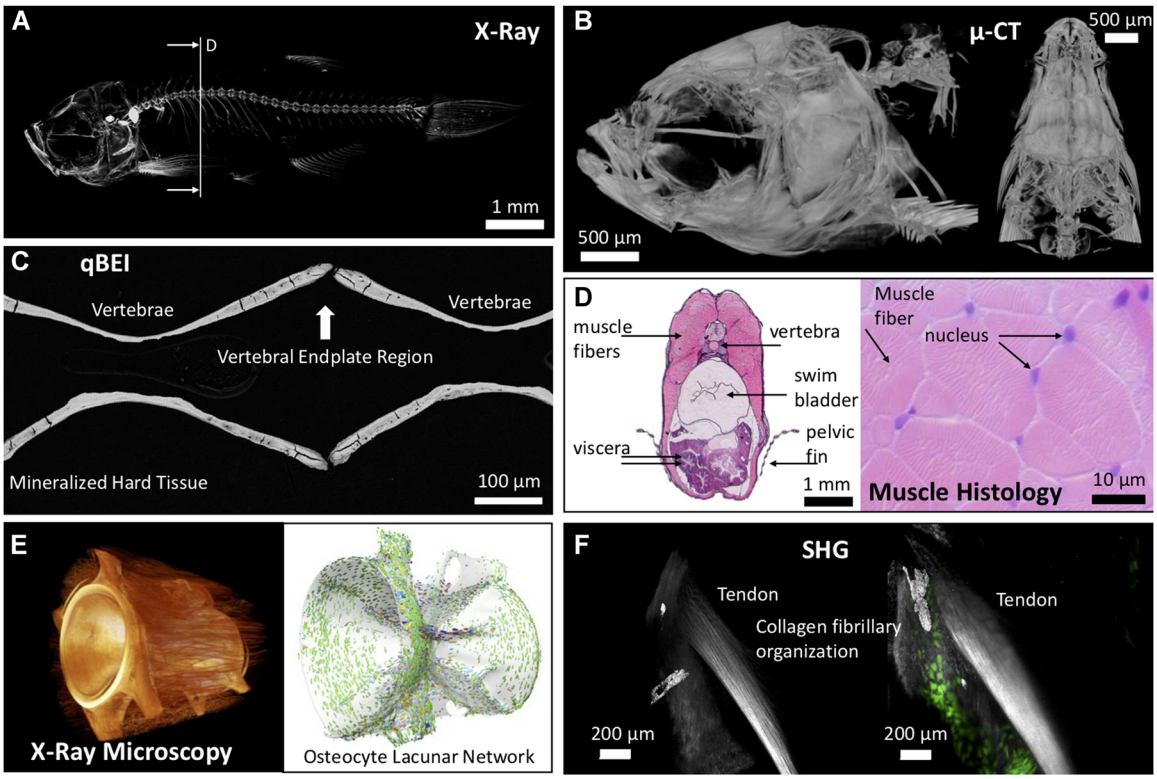

Figure 1. Imaging of tissue structure, composition, and quality. (A) Contact X-ray of a juvenile zebrafish. The vertical line shows the histological plane for the image in (D). (B) Microcomputed tomography (2 μm isotropic voxel size) of an adult zebrafish skull. (C) Quantitative backscattered scanning electron imaging (qBEI) in the spine of an adult zebrafish. Bone growth occurs at the vertebral endplates. (D) Hematoxylin and eosin (H&E) stained section of the zebrafish trunk. Muscle fiber density and cross-sectional muscle fiber area are readily assessed. (E) High-resolution imaging of a vertebral body via X-ray microscopy highlighting the osteocyte lacunar network. The osteocyte-lacunar orientation may reflect the orientation of collagen fibers, and loading patterns in zebrafish vertebrae. The lacunar orientation follows a specific pattern, that is, longitudinal orientation in the center of the vertebrae and circumferential orientation near the endplate regions. (F) An adult tendon attached to the maxilla in zebrafish is imaged using in vivo second harmonic generation (SHG) imaging, an indicator of type I collagen organization and density. The right panel shows dual imaging of tendon in concert with osteoblasts (green: osteocalcin+ cells expressing the *ocn:GFP* transgene). [Color figure can be viewed at wileyonlinelibrary.com]

studies suggest that retinoic acid is required for tendon cell condensation.[61,62] Recent studies have shown that mechanical force, through release of TGF-β, regulates the formation of tendon cell projections, which are thought to be involved in extracellular matrix (ECM) production.[31] In the adult, the cranial tendons have similar ultrastructure to mammalian tendons with highly ordered type I collagen fibrils observed by transmission electron microscopy.[45] In addition, they can be readily visualized using second harmonic generation (SHG) imaging (Fig. 1F).

Analogous to higher vertebrates, striated muscle of zebrafish contain three main components: contractile proteins, lipids, and connective tissue.[63] Vertebrae are connected by intervertebral ligaments.[64] Zebrafish possess both slow- and fast-twitch muscle fibers, which are topographically separated.[65] Together with cellular mineralized bone tissue, muscles, tendon, and other soft tissues, the zebrafish skeleton facilitates locomotion, provides mechanical support, and protects internal organs. In Figure 2, we compare the inter-vertebral space in the zebrafish and mouse as a case study of how skeletal structures in each species typically exhibit both morphophysiological similarities and differences.

Conservation of Developmental Programs

The molecules that govern zebrafish skeletal development are highly conserved with mammals. Sox9, a transcription factor necessary for chondrogenesis and skeletal development,[66] has two co-orthologs in the zebrafish, *sox9a*, and *sox9b*. They are expressed in overlapping and complementary patterns during development with *sox9a* in the pharyngeal arches and later restricted to the pre-chondrogenic mesenchyme that will form the jaw cartilage and fin scapulocoracoid, and with *sox9b* in the premigratory neural crest and fin endochondral disc.[7] The *sox9a* expressing chondrocytes also express *col2a1* and are Alcian Blue positive before three dpf. These skeletal elements will undergo perichondral or endochondral ossification and later become Alizarin red-positive cranial bones. In perichondral ossification, perichondral cells become *runx2a/b*, *osterix (sp7/osx)*, and *collagen 10* positive osteoblasts and initiate ossification.[67] Similar to other vertebrates, *indian hedgehog* co-orthologs (*ihha/b*) are expressed by chondrocytes and are thought to signal to patched, Hh receptors (*ptc1/2*) in the perichondrium and mediate bone formation.[68,69] Other cranial elements, such as the maxilla undergo direct intramembranous

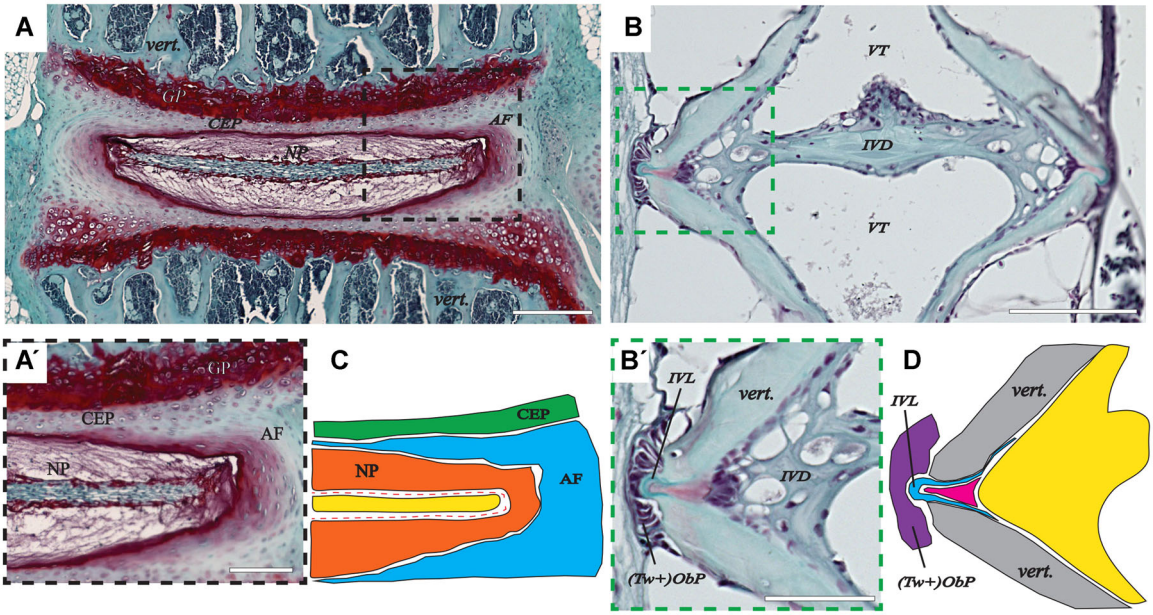

Figure 2. Comparison of the intervertebral disc (IVD) in mouse and zebrafish. (A-A′) and (B-B′): Midline section of a Safranin-O/Fast green staining of an intervertebral disc region in mouse (6 months) (A-A′) and zebrafish (1 year) (B-B′). (C) and (D): Cartoon schematic of insets for mouse (C) and zebrafish (D). In mouse, the IVD is composed of a proteoglycan-rich lamellar fibrocartilaginous cartilage called the annulus fibrosus (AF), which surrounds the nucleus pulposus (NP) joins adjacent bony vertebrae at the level of the cartilaginous endplate (CEP). Zebrafish IVD retains notochord-derived vacuolated cells embedded in a fibrocartilaginous matrix, however, there is no NP-like structure observed in zebrafish. An analogous structure to the outer AF layer is observed as a small acellular intervertebral ligament (IVL). Histologically, the NP in mouse appears to be composed of: an outer tissue layer, which stains for Safranin-O (orange in (C)); an inner cell layer (dotted red line in (C)); and an inner tissue layer that does not stain well for Safranin-O (yellow in (C)). In contrast, the zebrafish IVD has only weak Safranin-O staining (magenta in (B′, D)) in an interior region adjacent to the intervertebral ligament (IVL) (B′, blue in (D)). The zebrafish does not display a true cartilaginous NP tissue, rather the IVD is composed of vacuolated cells and fibrocartilaginous matrix. In contrast to mouse vertebrae, which contains bone marrow and trabecular bone filling the vertebrae, zebrafish vertebrae contain bone-shaped vacuolated tissue (VT). Twist-positive osteoblast progenitor cells ((Tw+)ObP) are observed adjacent to the IVL. AF, annulus fibrosis; CEP, cartilaginous endplate; GP, growth plate; IVL, intervertebral ligament; NP, nucleus pulposus; (Tw+)ObP, twist positive osteoblast progenitors; vert, vertebrae. [Color figure can be viewed at wileyonlinelibrary.com]

ossification via *osx*-expressing osteoblasts.[54,70] For many of these genes and cell types, reporter and lineage-tracing transgenic zebrafish lines have been generated, which, along with the optical access provided by zebrafish, allow unprecedented ability to visualize skeletogenesis (Fig. 3).

Physiology During Development and in Homeostasis

The skeleton serves as a key organ, which mediates systemic signaling affecting the physiology. Although many of these non-structural functions of the skeleton are just being identified, it is clear that many have conservation between humans and zebrafish. One key function of the skeleton is to facilitate mineral homeostasis. The skeleton participates in part by regulating phosphate homeostasis in the kidney through bone-kidney crosstalk. There is evidence that Fgf23, which in humans and mice, is synthesized in osteocytes and regulates kidney phosphate reabsorption, also regulates phosphate homeostasis in zebrafish.[71] In mammals, the skeleton also serves as a calcium and phosphorus reservoir. Because calcium regulation can occur through the gills in fish, compared with humans, the physiological role of the skeleton in calcium homeostasis in fish may differ.[53] Another function of the mammalian skeleton is acting as a site of hematopoiesis, as well as fat storage, in the marrow cavities. Zebrafish possess bone marrow spaces,[53] which is evident in endochondral bones, which are filled with fatty tissue.[54] However, unlike in humans, this is never colonized by hematopoietic stem cells (HSC). Thus, zebrafish bone marrow spaces lack hematopoietic tissue.[53] A number of zebrafish bones possess adipocytes within their marrow spaces,[54] however, it is unknown whether this adiposity responds to the metabolic demands, as it does in mice.[72] Finally, the skeleton can regulate the metabolic processes independent of mineral metabolism. For instance, the bone-derived hormone osteocalcin has been implicated in glucose homeostasis, cognition, and male fertility.[73] Whether the zebrafish skeleton functions as an endocrine organ through osteocalcin secretion requires further investigation.

Aging

Compared with early development, processes such as homeostasis and aging have not been studied in depth in the zebrafish. As certain debilitating conditions arise in the skeleton as a function of age, such as osteopenia and osteoporosis, the ability of the zebrafish to model components of these processes could be important. Zebrafish typically have a lifespan of approximately 2–3 years (though 5 years or more is possible),[74] and exhibit growth throughout life. There is evidence that zebrafish

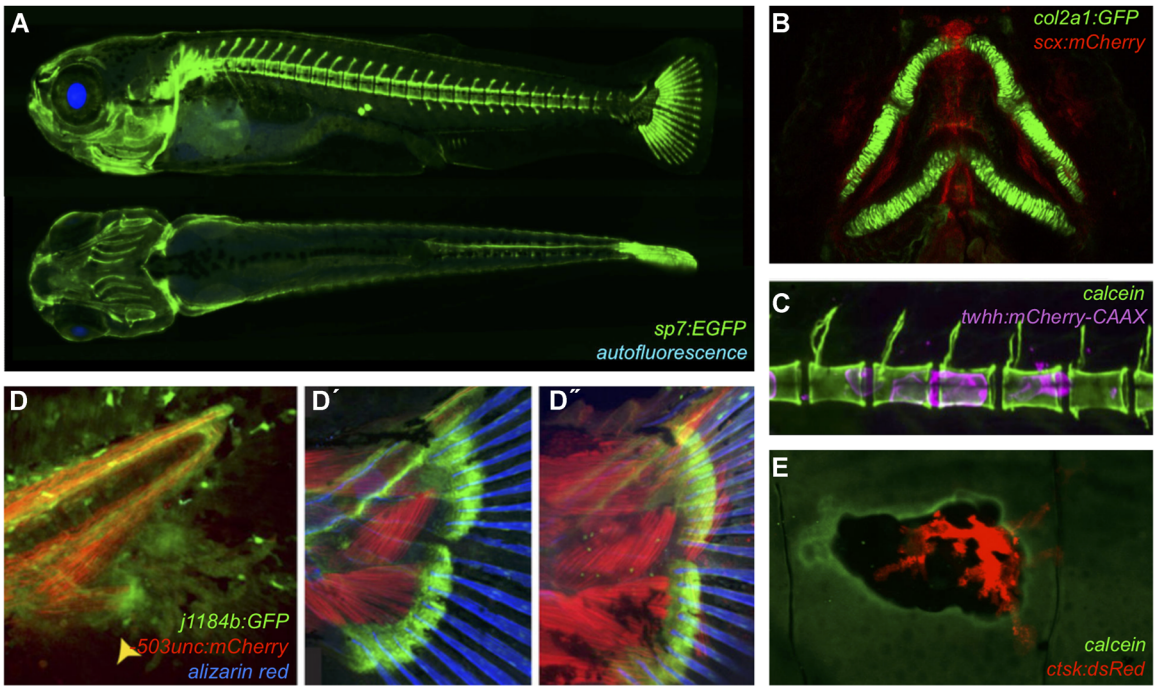

Figure 3. Live imaging of cell dynamics. (A) Whole-body images of a larval zebrafish (top: lateral view; bottom: ventral view), showing osteoblasts expressing osterix in the cranial skeleton, spine, and fins. (image source:[25]; use permitted under the Creative Commons Attribution 4.0 International License; image adapted from original). (B) Ventral view of three dpf zebrafish lower jaw showing *scxa:mCherry* expression in the forming tendon cells and *col2a1:eGFP* expression in chondrocytes. (C) Tol2-clonal notochordal expressing *twhh*-mCherry-CAAX cells (magenta) and spine marked by calcein (green) in 16 dpf zebrafish. (D) Representative imaging of caudal fin development demonstrating morphogenesis of the connective tissues (Tol2-EGFP^{j1184bGt}; green), muscle (*-503unc:mCherry*; red), and bone (Alizarin red—isolated with a far-red band filter; pseudocolored blue). (E) Osteoclast expressing *ctsk:dsRed* within resorption pit of an adult zebrafish scale stained with calcein to show mineralized matrix. [Color figure can be viewed at wileyonlinelibrary.com]

skeletal function declines with age. For instance, tendon mechanical properties diminish with age.[75] Moreover, alterations of vertebral bone and disc are observed in aged zebrafish.[76] The bone dependence on estrogen has been modeled in another small teleost, medaka, and thus the basic properties of the etiology are likely present in zebrafish.[77] With more analysis of late developmental stages, it is likely more insight will emerge from the zebrafish into how the skeletal system ages and its consequences.

Regeneration and Repair

Zebrafish have not been used as a common model for understanding human fracture repair. This is in part due to lack of accessible long bones, as well as its high regenerative capacity, which may utilize different repair mechanisms than in mammals. Previous studies have examined the repair properties of damaged membranous bones of the skull roof [78] as well as mandible.[79] Although these are not directly comparable with analysis of long bone fractures studied in mouse and most commonly seen in patients, there were some similarities in terms of the genes and cell types involved. For example, *runx2+* cells in the periosteum were likely involved in new bone formation and proper formation of the cartilage callus relied upon *Indian hedgehog a* (*ihha*).[79] In addition to the examination of intrinsic regenerative mechanisms, the transparency of the zebrafish permits the analysis of extrinsic cell populations in the healing process. Studies have shown that the immune system plays an important role in mediating tissue regeneration.[80,81] Visualization of immune infiltration after injury can be accomplished through the use of transgenic reporter lines that either label all leukocytes (*cd45:DsRed*)[82] or are specific for neutrophils (*mpx:GFP*^{i114Tg})[83] or macrophages (*mpeg:eGFP*).[84] There are also several methods to functionally deplete immune cell populations (reviewed in Keightley et al.[80]), which can permit temporal control over cell type-specific cell ablation to assess the role of immune cell populations at different stages of the regenerative process, as has been performed for tail fin regeneration.[85]

Zebrafish have a significant capacity for epimorphic regeneration.[3,14] One example is the caudal fin, which regenerates following amputation.[86] Similar to salamander limb regeneration, fin regeneration involves a heterogeneous pool of progenitors called the blastema, which is comprised, at least in part, of mature cells at the amputation stump that dedifferentiated, including osteoblasts.[36] A variety of pathways known to be important for skeletogenesis in mammals are recapitulated during fin redevelopment, as reviewed in Watson et al.[86] An intact musculoskeletal system is required for normal regeneration as zebrafish subjected to injection of botulinum toxin, which inhibits synaptic release at cholinergic nerves, exhibit impaired regeneration.[87] This model has also revealed the existence of mesenchymal progenitor

populations within specific regions that robustly respond to injury and generate new *osx*+ osteoblasts.[88,89] Dedifferentiation of mature osteoblasts also occurs during repair of zebrafish fin fractures and skull injuries.[78] While osteoblast dedifferentiation is more limited in mammals, fin repair after fracture exhibits some similarities to mammalian long bone fracture, including formation of a remodeling callus,[90] and recruitment of osteoclasts.[91] Recently, it was shown that neutrophils dynamically colonize the fracture site. When infected with *Staphylococcus aureus*, neutrophils were retained in the fracture site and repair was reduced.[91] Further studies examining the utility of the fin fracture model to study the aspects of fracture biology are warranted.

Musculoskeletal Loading

While the zebrafish skeleton has a reduced role in resisting gravitational loads relative to humans, there is evidence that the zebrafish skeleton can respond to exercise, as well as disuse. Swim training routines have been established to force exercise and stimulate natural modes of skeletal loading in zebrafish. In this way, the complex interplay of cellular, structural, and compositional bone characteristics can be assessed using multiscale approaches in zebrafish to study the effects of genetic and environmental interactions on the skeletal system in vivo. During early development in zebrafish, swim training alters the timing of skeletogenesis.[92] In adult zebrafish, swim training increases vertebral bone formation and alters quality.[58] Moreover, this type of forced exercise also induces muscle adaptations in adult zebrafish.[93] This paradigm opens up avenues for genetic and small molecule screens to identify signaling pathways critical for musculoskeletal adaptation to loading and exercise.

PHENOTYPING

MicroCT

The three-dimensional (3D)-high-resolution microcomputed tomography has become established as a powerful method to assess bone morphology and microstructure in zebrafish.[18,58,94,95] Using a 5 μm voxel size, bone structure indices as vertebral bone volume, thickness, and eccentricity can be characterized.[18,58] Neural arch area, which reflects modeling arising from osteoblast and osteoclast activity, can also be captured.[94] Because of their small size, whole body, high-resolution scans are readily acquired.[95] Software for semi-automated segmentation enables in-depth phenotyping at a large number of skeletal sites. By quantifying hundreds of measures this was shown to increase the sensitivity in discriminating mutant populations.[95] Moreover, the osteocyte lacunar network in the vertebral tissue can be imaged at high resolution with lab-based nano-CTs and 3D X-ray Microscopy (3DXRM). The orientation of the osteocyte lacunae in relation to the long and short axis of the vertebral bodies, sphericity, mean lacunar volume, and lacunar density can be quantified.[18,58] Finally, synchrotron-based X-ray microCT, when combined with tissue-contrast stains, can yield whole-organism images suitable for cell-level quantitative histological phenotyping in zebrafish.[96]

Histomorphometry

In zebrafish, histologic sections stained using von Kossa/van Gieson, Goldner's modified Masson-trichrome, and toluidine blue enable static bone histomorphometry, and is performed in accordance with standardized nomenclature set forth by the ASBMR nomenclature committee for practitioners of bone histomorphometry.[97] Calcein labeling or double labeling with calcein and Alizarin Red S can be performed and double labels can be evaluated for dynamic bone histomorphometry.[18,58] Such an approach was used to quantify increases in mineral apposition rate (MAR), mineralizing surface per bone surface (MS/BS), and bone formation rate (BFR) at the vertebral endplates in zebrafish subjected to swimming exercise.[58]

Assessment of Bone Composition, Mineral Density Distribution, and Mechanical Properties

Recently, quantitative backscattered electron imaging (qBEI) has been established as an effective means to measure the bone mineral density distribution in zebrafish.[18,58] Gray value histograms were used to assess the mean calcium content in the mineralized bone tissue, as well as the homogeneity of mineralization.[18,58] Vibrational spectroscopy methods (e.g., Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy) have also been adapted to zebrafish bone.[18,98] Parameters such as the mineral-to-matrix-ratio, carbonate-to-phosphate ratio, cross-link-ratio (collagen maturity), and crystallinity (purity, size of mineral crystals) of the bone were shown to provide information about the molecular and compositional bone characteristics.[18] Finally, nanoindentation of vertebrae can be performed in zebrafish to assess the local mechanical and material properties such as Young's modulus (elastic modulus), hardness, and fracture toughness.[18] The biomechanical properties of zebrafish cranial tendons can also be measured. A maxillary tendon was found to have stress–strain nonlinearity and a linear modulus similar to mammalian tendon data.[75]

DISEASE APPLICATIONS

Collagenopathies

OI is a disease of the collagen matrix, which results in brittle bones and skeletal deformities. In humans, collagen type I is a heterotrimer composed by two α chains, α1(I) and α2(I), which trimerize in a 2:1 ratio, respectively, to form a fibril with a triple-helix structure. In zebrafish, the collagen type I triple helix is composed of three α chains, α1(I), α2(I), and α3(I), which are encoded for by the genes *col1a1a*, *col1a2*, and *col1a1b*, respectively.[99] Most human patients with OI are attributed to mutations in type I collagens, with the

majority of mutations disrupting the conserved Gly-X-Y motifs responsible for fibrillar assembly of the collagen heterotrimers.[100] In zebrafish, several dominant mutants have been identified carrying heterozygous glycine substitution in the α1 chain of collagen type I, and which exhibit severe, pathological features of classical OI. This was demonstrated in the *chihuahua* mutant, which exhibited changes in vertebral tissue composition.[18] A large panel of zebrafish mutants of *col1a1* genes with qualitative and quantitative defects in collagen type I have been characterized, and found to mirror genotype–phenotype relationships of the range of OI subtypes found in humans.[16–20] Disease models have also been developed for mutations affecting COL1A2[17] as well as rare recessive forms of OI affecting non-collagenous proteins (e.g., PLOD2[95,101] and BMP1[95,102]).

Spinal Curvature

Adolescent idiopathic scoliosis (AIS) is defined as scoliosis without underlying vertebral malformations.[103] While the pathogenesis of this disease is still controversial, the zebrafish has made significant advances in our understanding of this disorder. AIS-like scoliosis was demonstrated in *cc2d2a* mutant zebrafish, which has a role in vesicle trafficking and fusion at the transition zone of photoreceptor connecting cilium in the eye.[104] Recently, mutant zebrafish displaying larval or late-onset scoliosis without vertebral malformations, analogous to AIS, have been described for *c21orf59*, *ccdc40*, *ccdc151*, *dyx1c1*, *kif6*, and *ptk7*.[105,106] A common mechanism has emerged from these studies, where loss of ependymal cell cilia function lining the ventricles of the brain, leading to reduced cerebrospinal fluid flow can generate AIS in zebrafish.[105,107] Interestingly, maternal-zygotic *ptk7* mutant zebrafish display scoliosis with vertebral malformations, while strictly zygotic *ptk7* mutant fish display late-onset AIS without vertebral malformations.[108] This suggests that the severity of scoliosis can be on a spectrum based on temporal requirements for gene function, which may explain the strong association of AIS in families of children with CS.[109]

The cellular mechanism of AIS in *ptk7* was demonstrated by a *foxj1:ptk7* transgenic zebrafish, which can completely rescue the onset of scoliosis phenotypes observed in *ptk7* mutant zebrafish.[105] Foxj1 is a master transcriptional regulator of motile cilia,[110] which labels motile cilia of the ventricles of the brain and in the pronephros but also labels a subset of central canal lining ciliated cerebrospinal fluid (CSF)-contacting neurons. Indeed, disruption of a major signaling receptor, *pdk2l1*, of CSF-contacting neurons led to mild alterations in spine curvature in zebrafish.[111] While it is still unclear how these disruptions of CSF physiology cause scoliosis, recent studies demonstrated that CSF flow (i) helps to stimulate the proper formation of the extracellular Reissner's fiber, which can directly contribute to body straightness during embryonic development, via an unknown mechanisms[112]; and (ii) that CSF flow transports adrenergic signals, which stimulate the expression of urotensin neuropeptides from CSF-contacting neurons along the spinal cord and mutant zebrafish of the urotensin receptor *uts2ra* display AIS.[113] How these studies will translate to mammalian physiology is still unclear. However, at least one candidate gene for AIS uncovered in zebrafish *kif6*[106] does not recapitulate AIS phenotypes when mutated in mouse or human.[107]

Although having early differences in vertebral specification compared with mammals, the zebrafish may also serve as a model for aspects of congenital scoliosis (CS). Disruption of the extracellular sheath through chemical disruption of lysyl oxidases [114] or specific genetic disruptions of the sheath ECM components of the notochord sheath such as: *col8a1a*, *col27a1a/b*, or *calymmin*[16,115,116] can generate CS-like scoliosis with vertebral malformations in zebrafish suggesting potential underlying components of zebrafish development that can be used to assess gene function in CS etiology.

Disease Loci

Human genome-wide association studies (GWAS) are a powerful means to understand the genetic risk factors for chronic diseases such as osteoarthritis[117] and osteoporosis.[118] These loci may harbor novel drug targets for orthopedic diseases, as evidenced by the fact that OPG/RANK/RANKL and LRP5/SOST, all genes at BMD loci, are members of pathways targeted by osteoporosis drugs (Denosumab and Romosozumab, respectively). A recent analysis of UK-Biobank data identified 515 loci associated with eBMD.[118] The causal genes responsible for most associations have yet to be assigned, and thus gene discovery in animal models is needed to complement GWAS in human populations.[11] One limitation with knockout mice is the lack of coverage of genes at BMD loci. For instance, of the >23,000 protein-coding genes in the mouse genome, <5% have been rigorously analyzed for bone phenotypes in knockout mice within ancillary bone phenotyping projects of the International Mouse Phenotyping Consortium.[11] A recent study demonstrated the potential to rapidly generate mutations in CRISPR-edited G0 zebrafish to attribute functional contributions of candidate genes at bone disease loci.[25] Skeletal abnormalities have been observed in fish with altered function in other genes at BMD loci, including LRP5, OSX/SP7, and RANKL.[119–121] This provides further evidence that the identification of genes that contribute to human osteoporosis-related traits in zebrafish is feasible. The utility of zebrafish for human skeletal genomics is reviewed in Kwon et al.[11]

Osteoarthritis

Recent data has shown that zebrafish can be used to study the development of synovial joints and

osteoarthritis. Mutations in *col11a2* in zebrafish leads to joint pathologies reminiscent of early-onset osteoarthritis in humans.[122] Further, as in humans and mice, zebrafish lubricin or *proteoglycan-4b* (*prg4b*) expression was found within some joint regions, such as articular chondrocytes in the jaw.[123] These joint regions also expressed *col10a1*, *acana*, and *matrilin1*, which together with *prg4*b, showed similarities to genes expressed in mammalian synovial joints. Loss of both *prg4* orthologs resulted in synovial hyperplasia and deterioration of the joint surface by 6–12 months of age. This phenotype recapitulates that found in mouse where loss of Prg4 results in joint disease by 2 months.[124] Although it is unknown why there is a significant delay in timing of osteoarthritis onset in the zebrafish compared with mouse genetic models, possible explanations are the zebrafish's robust regenerative abilities combined with different mechanical environments. Even with these differences, this work establishes the conservation in synovial joint gene expression and function in the zebrafish.

SUMMARY/FUTURE DIRECTIONS

The genetic causes of skeletal disorders are rapidly becoming identified, and it is now clear that many common musculoskeletal disorders are fundamentally complex in their causes.[118,125] The field is faced with the need to more fully interrogate the functional consequences of genetic and environmental stresses in how the skeleton and its connecting tissues are formed, how it integrates with broader physiology, and how it repairs the damage. There is now a strong rationale to validate newly discovered disease candidate genes in the zebrafish prior to extensive mouse analyses. The refinement of clonal analyses of gene function will expedite the combinatorial analysis of gene function and permit a systematic testing platform for genetic association studies and analysis of genetic modifiers. With the ability to visualize cellular dynamics during the formation of the skeleton as well as during repair, in different genetic contexts, the zebrafish provides a powerful system to bring functional characterization in line with the rate of genetic discoveries.

In addition to validation, the zebrafish is also a valuable platform for discovery. Due to its small size, low cost, and genetic malleability, the zebrafish has opened new screening methods that have already discovered small molecules efficacious in regulating skeletal phenotypes. Similarly, through unbiased mutational screening, new genes—and new functions for existing gene—have been discovered. These mutations have shown to be predictive of causes of skeletal disorders in humans,[126,127] and opened new areas of musculoskeletal development previously uncharacterized or neglected.[113,128]

As the use of zebrafish for orthopedic research is still in its relative infancy, there are a number of open questions regarding the developmental stages, bones, and phenotypic traits in zebrafish that best serve as a model for human skeletal biology.[11] Morphophysiological differences can make one-to-one modeling of human skeletal phenotypes in zebrafish challenging. Origins of mammalian bones and their connections to fish bones (e.g., the mammalian middle ear bones, which derive from bones that form the jaw in fish[129]) can sometimes be revealed through evolutionary analyses, however, such relationships cannot always be made. In this context, a community effort to phenotype zebrafish mutants for orthologs of genes examined in mutant mouse phenotyping consortiums may aid in identifying zebrafish phenotypes that are most consistently associated with phenotypic changes in the orthologous mutant mouse.[11]

With the extension of zebrafish work into phenotypes of the skeleton beyond early development, the broad utility of this model has emerged. While there are differences stemming from the use of a non-mammalian vertebrate for modeling human disorders, the zebrafish model provides both genetic and anatomical foundations, in which informed analyses can be made concerning the etiology of disorders and also serves as a tool to refine potential therapeutic strategies.

AUTHORS' CONTRIBUTION

B.B., J.L.G., R.S.G., M.P.H., and R.Y.K. drafted and revised the manuscript. All authors have read and approved the final submitted manuscript.

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